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ABSTRACT

A collaborative project between the International Rice Research Institute (IRRI) and the Rural Development Administration (RDA) of Korea was initiated in 1983 with the objective of developing cold-tolerant lines through anther culture of F_1 hybrids. The scheme included hybridization work at RDA and IRRI, anther culture of the hybrids at IRRI, and collaborative screening, selection, and field testing by RDA and IRRI of the lines obtained.

By December 1985, 8,670 anther culture-derived plants had been produced, of which 3,617 (41.7%) were autodiploids, and 1,270 progenies had been sent to Korea for screening for cold tolerance.

Callus induction efficiency was dependent on genotype, although all crosses tested were amenable to anther culture. Addition of glucose to the medium was required for higher efficiency during wet season but seemed detrimental during dry season. Green plant regeneration, which was higher during dry season, was enhanced by culturing the calli in plant regeneration medium containing 10-30 mg abscisic acid/liter.

Preliminary tests of the lines for cold tolerance at the seedling stage showed that some were more tolerant than either parent, indicating the possibility of isolating a better cold-tolerant variety from these materials.

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IRRI-KOREA COLLABORATIVE PROJECT FOR THE DEVELOPMENT OF COLD-TOLERANT LINES THROUGH ANTHER CULTURE

Rice is the major source of calories for 40% of the world's population. Although second to wheat in area harvested, rice ranks first as a food crop, providing more calories per hectare than any other cereal (3).

Because arable land is becoming scarce, less favorable environments are mandated for rice production. Most of the world's rice is grown in the tropics, and the critical determinant for growth appears to be temperature (3). For example, in South and Southeast Asia alone, modern rice varieties cannot be planted on 7 million ha because of cold water and cold weather — the two factors that cause cold injury (10). Low temperature affects the growth of rice from seeding to the reproductive stage (4), with the booting stage the most sensitive (16). In some areas, two rice crops per year would be possible if cold-tolerant varieties were developed (2).

Breeding for varieties with increased cold tolerance is difficult because of several factors. Highly cold-tolerant varieties have unfavorable genetic correlations with other agronomic characters, e.g., lodging resistance, grain quality, disease resistance, and yielding ability (13). In breeding programs in Taiwan, the emphasis is on improved cold tolerance and disease resistance for the indica type. Japonica/indica crosses have been made and backcrossed to japonicas to enhance the cold tolerance of the indicas. Such crosses have shown high degrees of sterility (8). Based on current knowledge, cold tolerance is a complex, polygenic trait (9). Some varieties that have consistently shown a high degree of cold tolerance and blast resistance are poor combiners (10). There is also difficulty in screening the early generation progeny of japonica/indica crosses for cold tolerance because of the presence of partially sterile segregants. In some cases, sterility may be due to intersubspecific hybridity rather than to cold susceptibility (10).

An alternative to conventional breeding in the production of cold-tolerant lines is anther culture. With anther culture of F_1 hybrids involving one or both cold-tolerant parents, homozygosity is immediately fixed, and difficult-to-recover plant characters can be obtained.

To test the applicability of this technique to breeding for cold tolerance, a collaborative project between IRRI and the Rural Development Administration (RDA) of Korea was established (Fig. 1). Because it is necessary to regenerate as many plants as possible, knowledge from our preliminary studies on increasing the efficiency of callus production and plant regeneration was applied to the anther culture of F_1 sexual crosses.

We investigated the effects of genotype, season, and glucose on callus induction and of genotype, season, and abscisic acid on plant regeneration. We also evaluated the cold tolerance of some of the regenerated lines and their parents.

MATERIALS AND METHODS

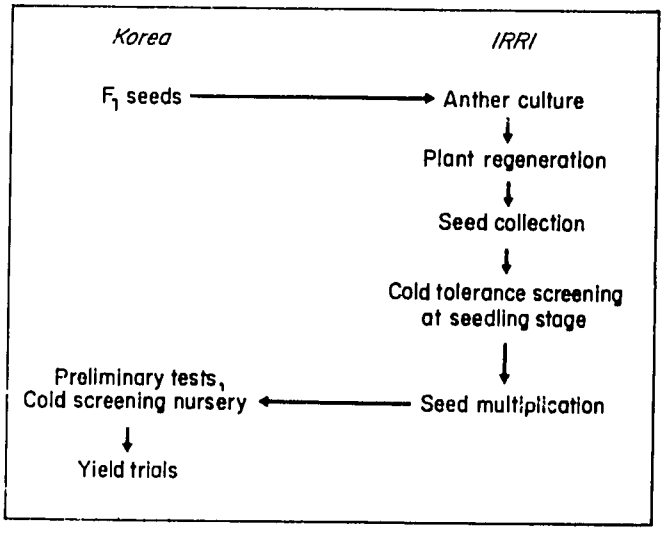
Test materials

Seeds of 23 crosses (Table 1) made at RDA were grown in the IRRI screenhouse for panicle collection. The parents were selected mainly for their cold tolerance and blast resistance. Each F_1 seedling was sown in a plastic pot containing about 1 kg of soil supplied with 1.5 g $(NH_4)_2(SO_4)$, 0.5 g $Ca(H_2PO_4)_2$, and 0.5 g KCl. Panicles were collected when the distance from the auricle of the flag leaf to that of the subtending leaf was 5-10 cm.

Callus induction

Method. Panicles from the 23 crosses were sterilized in a 20% solution prepared from 5.25% NaOCl stock (commercial bleach) for 30 min.

The panicles were divided into the upper and lower halves, and a floret from the middle of each half was fixed in a solution of 3 parts ethanol:1 part acetic acid to which 2% $FeCl_3 \cdot 6H_2O$ was added as a mordant. The stage of pollen development was determined from these samples using 2% acetocarmine stain. In the meantime, the rest of the anthers were subjected to cold treatment (8 °C for 8 d).



1. IRRI-Korea collaborative program for the production of cold-tolerant lines through anther culture.

Table 1. Parentage of F_1 hybrids for cold tolerance.

Cross	Parents
SR11191	SR10249-B-B/Baegyangbyeo
SR11193	SR10249-B-5/Milyang 55
SR11194	SR10249-B-5/SR8102-127-4
SR11238	Milyang 54/IR20654R5-15-1
SR11242	SR8012-127-4/IR20654R5-15-1
SR11426	Fukuhikari/Fukei 126
SR11428	Fukuhikari/Fujihikari
SR11433	Chulweon 32/Fujihikari
SR11436	Reimei/Fujihikari
SR11440	Seolagbyeo/Fujihikari
SR11451	RAC3/Hamaasahi
SR11452	RAC3/Ishiokamochi
SR11453	RAC3/Fujihikari
SR11455	Suweon 303/Hamaasahi
SR12192	Milyang 64/Sinseonchalbyeo
SR12194	Milyang 64/Seomjinbyeo
SR12196	Akibare/Seonambyeo
SR12200	Mineyutaka/Seomjinbyeo
SR12211	Norin 6/SR4084-5-4-4-1
SR12212	Norin 6/Seonambyeo
SR12246	54 Bt-68/Seonambyeo
SR12259	Chukaka/Seonambyeo
SR12291	Seonambyeo/Seomjinbyeo

Only anthers at the miduninucleate to early binucleate stages of development were inoculated in liquid E-24 medium modified from Gamborg's B5 medium (17). The cultures were incubated in dim light at 25 °C. Data on callus production were collected after 6 wk.

Effect of season and glucose on callus induction. Some F_1 crosses were grown during 1983 wet season (WS) and 1984 dry season (DS) to study the effect of season and glucose on callus induction. The plants were grown in the screenhouse. The same cultural practices were maintained in both seasons; only the environment varied.

The anthers were plated on two media: E-24 and G4, which is E-24 without glucose (Appendix). The procedure for anther inoculation was as previously stated.

Plant regeneration

Method. The calli on E-24 were transferred to M-shaped paper bridges positioned inside test tubes containing 10 ml of liquid N-19 medium (Appendix) (18). The calli were incubated at 1000 lux in 9/15 h light/dark conditions daily at 25 °C. After 4 wk the calli were transferred to semisolid N-19. Data on plant regeneration were collected after another 4 wk.

Effect of season. The calli plated on E-24 for 1983 WS and 1984 DS were plated following the same procedure.

Effect of abscisic acid. Several F_1 crosses that had high callus production were used. Calli from E-24 were transferred onto paper bridges with the following concentrations of abscisic acid (ABA) using N-19 as a basal medium: N-19, 0 mg ABA/liter; MSA_5, 5 mg; MSA_{10}, 10 mg; MSA_{15}, 15 mg; MSA_{20}, 20 mg; MSA_{25}, 25 mg; and MSA_{30}, 30 mg.

The incubation conditions were the same as previously stated. Calli from all treatments were transferred to semisolid N-19 4 wk after ABA treatment.

Screening for cold tolerance at seedling stage

The regenerated plants were grown in the IRRI Phytotron until maturity. Seedlings of the progeny of regenerated plants were screened for cold tolerance by the IRRI Plant Physiology Department. Ten seeds from each line and their corresponding parents were sown in enamel trays and grown in the screenhouse until they reached the 3-leaf stage. The seedlings were then set in a water bath at 12 °C. The plants were scored for cold tolerance 10 and 13 d later (15) on a 1-9 scale: 1 = seedlings dark green, 3 = seedlings light green, 5 = seedlings yellow, 7 = seedlings brown, 9 = seedlings dead.

Screening of anther culture-derived lines in cold tolerance nursery

To measure the efficiency of anther culture for cold tolerance breeding, 22 anther culture-derived lines of IR crosses that showed cold tolerance at the seedling stage were screened in the cold tolerance nursery at the Chuncheon Research Center of RDA, Korea. The crosses were made by the IRRI Plant Breeding Department, and each cross had at least one cold-tolerant parent. The field had an increasing water temperature gradient from the inlet (17 °C) to the outlet (25-27 °C). Susceptible and resistant check varieties were included for comparison. The plants were grown to maturity, and data on agronomic characteristics were recorded.

RESULTS AND DISCUSSION

Callus induction

Effect of genotype. Variation in culturability among the 23 F_1 crosses was evident (Table 2). Callus induction percentage ranged from 0.5 to 54.5. Genotype thus has a strong influence on amenability to anther culture techniques. Genotype as a factor in inducing callus production has been previously reported in rice (6). Ongoing studies using reciprocal crosses may reveal whether callus induction efficiency is cytoplasmic or genic.

Effect of season and glucose. Interactions between seasons and between the presence and absence of glucose in the medium were observed (Table 3). Anthers tended to be unsuitable when F_1 plants were grown under short-day conditions, which in this case was during WS (11). However, supplying a readily available C source in the form of glucose increased callus induction efficiency in all but one of the crosses (SR11455). Because WS days are short and light intensity is often limiting, the available carbohydrates that accumulate in the plant may be lower than the optimum level C source that must be supplied to the pollen.

On the other hand, adding glucose to the culture medium for callus induction of anthers from DS was detrimental. Supraoptimal concentration of carbohydrates (endogenous plus exogenous in the form of sucrose and glucose) may have suppressed callus formation.

Other substances such as endogenous hormones, the production of which is greatly influenced by environmental factors, could also have influenced subsequent callus induction in *in vitro* cultures.

Table 2. Effect of genotype on callus induction using E-24 liquid medium, 1983 WS.

Cross	Anthers plated (no.)	Calli produced	
		no.	%
SR11191	364	2	0.5
SR11193	743	7	0.9
SR11194	745	11	1.5
SR11238	1,036	44	4.2
SR11242	366	7	1.9
SR11426	677	369	54.5
SR11428	539	218	40.4
SR11433	2,079	434	20.9
SR11436	663	117	17.6
SR11440	643	242	37.5
SR11451	2,508	893	35.6
SR11452	2,192	515	23.5
SR11453	1,504	488	32.4
SR11455	474	52	11.0
SR12192	1,569	254	16.2
SR12194	503	10	2.0
SR12196	1,520	313	20.6
SR12200	1,022	119	11.6
SR12211	1,185	321	27.1
SR12212	523	270	51.6
SR12246	481	148	30.8
SR12259	510	105	20.6
SR12291	147	21	14.3
Total	21,993	4,960	
Av	956.2	215.6	22.6

Table 3. Percentage of callus production[a] in medium with glucose (E-24) and without glucose (G4), 1983 WS and 1984 DS.

Cross	1983 WS		1984 DS	
	G4	E-24	G4	E-24
SR11426	0	29.7	39.4	54.5
SR11428	5.6	41.7	63.1	40.4
SR11433	11.6	81.2	46.4	20.9
SR11436	40.4	53.2	27.2	17.6
SR11440	0.8	13.2	55.1	37.5
SR11451	0.8	28.2	40.3	35.6
SR11452	15.1	101.8	19.2	23.5
SR11453	1.7	84.0	39.3	32.4
SR11455	11.2	5.9	13.1	11.0
Av[b]	9.0	48.6	37.0	29.5
Av for season	33.7		33.2	

[a] $\frac{\text{No. of calli produced}}{\text{No. of anthers plated}} \times 100$. [b] $\frac{\text{Total no. of calli produced}}{\text{Total no. of anthers plated}} \times 100$.

Table 4. Effect of genotype and season on plant regeneration with callus induction medium E-24 and regeneration medium N-19.

Cross	1983 WS		1984 DS	
	Calli responding (%)	Av green plant production (no.)	Calli responding (%)	Av green plant production (no.)
SR11433	0	0	6.8	15.7
SR11436	0	0	27.3	7.7
SR11451	7.7	2.0	21.8	1.5
SR11452	16.2	7.2	29.8	4.8
SR11453	26.1	6.0	23.1	6.7

Table 5. Effect of abscisic acid on plant regeneration, 1984 DS.

Cross	N-19	MSA_5[a]	MSA_{10}	MSA_{15}	MSA_{20}	MSA_{25}	MSA_{30}
	Percentage of calli responding						
SR11433	6.8	2.7	5.7	2.8	21.2	9.4	6.7
SR11451	21.8	9.6	34.0	27.4	25.5	34.6	81.6
SR11452	29.8	34.8	36.5	29.4	36.7	43.8	38.8
SR11453	23.1	12.8	18.2	19.5	19.5	13.5	18.9
SR12192	5.3	13.3	15.8	11.8	23.5	18.8	18.8
Av[b]	19.8	15.8	25.0	20.4	26.2	27.0	39.2
	Average plant production						
SR11433	15.7	4.0	19.0	1.0	8.4	7.7	23.0
SR11451	1.5	8.2	5.1	4.1	7.0	5.2	4.0
SR11452	4.8	5.5	6.7	7.8	6.8	7.1	6.6
SR11453	6.7	7.0	11.5	7.0	12.2	21.6	8.7
SR12192	4.0	8.0	18.0	1.5	11.8	2.7	3.3
Av[c]	5.1	6.3	8.1	5.9	8.4	7.6	5.7

[a] MSA = Murashige-Skoog medium with abscisic acid added at indicated mg/liter.

[b] $\frac{\text{Total no. of calli responding}}{\text{Total no. of calli plated for regeneration}}$.

[c] Plants produced/calli producing plant.

Plant regeneration

Effect of genotype and season. Genotypes varied in their effect on plant regeneration in different seasons (Table 4). Anthers of F_1 plants grown in DS were generally more responsive than those grown during WS. Better response of plants grown in DS could be because of lower incidence of insect pests and diseases, thus healthier and better inoculum (anthers). During DS, photosynthetic activity is higher because of increased solar radiation, and therefore the substances for active growth such as sugars and endogenous growth substances could be present in adequate quantities.

Effect of abscisic acid. The percentage of calli responding to ABA treatment increased with the addition of ABA to the preculture medium (Table 5). Although the genotypes varied in response, ABA increased plant production, with an optimum concentration distinct for each cross. On the average the percentage of calli responding was highest at around 20-30 mg ABA/liter, while average plant production per responding calli was highest at 10-20 mg/liter.

The stimulatory effect of ABA on plant regeneration could be through its influence on proline accumulation (12). Proline is a readily available source of C and reduced N, and stimulates xylary element formation (1). These results agree with those obtained from somatic rice callus (5) and anther-derived rice calli (14).

Cold tolerance screening of anther culture-derived lines at seedling stage

Variability was observed in the response to cold tolerance of various lines of the same cross (Table 6), showing that different recombinants from the same cross were obtained and expressed. In the F_1 cross RAC3/Ishiokamochi (SR11452), 6 of 278 anther culture-derived lines exhibited increased tolerance (10-d treatment) relative to both parents. Increase in tolerance of the anther culture-derived lines was more pronounced when the seedlings were scored 13 d after

treatment, when 157 lines (56.5%) were more tolerant than either parent. Increase in tolerance of the anther culture-derived lines could be due to synergistic, additive, or complementary effects of genes from parents.

Screening of anther culture-derived lines in cold nursery

Among the 22 crosses tested, 3 promising ones were identified to be better than the resistant check in some characteristics (Table 7). For example, two of the lines had higher fertility percentage at the outlet, while the other had a value similar to the resistant check. The differences in fertility percentage of 1166 and 1166-4 (japonica/indica crosses between Tatsumi mochi and BG90-2) at the inlet and outlet were less than that of the resistant check, suggesting that fixation of a character is achieved through anther culture. Also, the three promising anther culture-derived lines proved to be more vigorous than the resistant check.

Total plant regeneration

From 1983 WS until end December 1985, 8,670 plants that could be considered potential lines were regenerated (Table 8). Of these, 3,617 (41.7%) were autodiploids, and therefore produced seeds. The rest were either haploids, polyploids, or aneuploids, or died from stress during transfer from aseptic to normal growing conditions.

By December 1985, 1,270 lines had been sent to RDA for screening in the cold tolerance nursery, while the rest were being multiplied at IRRI. From the number of plants regenerated, we are optimistic that a line will be selected for release as a good breeding line or variety.

Anther culture could likewise be applied to the production of homozygous lines from F_1 hybrids that are produced for other objectives.

Table 6. Cold tolerance score at seedling stage of anther culture-derived lines (first-generation seeds from regenerants were used) and respective parents.

Cross	Parents	Lines tested (no.)	Lines (no.) with a score of[a]				
			1	3	5	7	9
	After 10 d at 12 °C						
SR11453	RAC3/Fujihikari	262	0	244	18	0	0
	RAC3			x			
	Fujihikari		x				
SR11452	RAC3/Ishiokamochi	278	6	239	33	0	0
	RAC3			x			
	Ishiokamochi			x			
SR11451	RAC3/Hamaasahi	27	0	11	16	0	0
	RAC3			x			
	Hamaasahi			x			
SR11426	Fujihikari/Fukei 126	1	0	1	0	0	0
	Fujihikari		x				
	Fukei 126		x				
SR11433	Chulweon 32/Fujihikari	39	16	23	0	0	0
	Chulweon 32		x				
	Fujihikari		x				
	After 13 d at 12 °C						
SR11452	RAC3/Ishiokamochi	278	2	155	102	16	3
	RAC3				x		
	Ishiokamochi				x		
SR11433	Chulweon 32/Fujihikari	39	0	19	20	0	0
	Chulweon 32			x			
	Fujihikari			x			

[a] x = tolerance level of parent.

Table 7. Promising anther culture-derived lines in cold tolerance tests conducted in Chuncheon, Korea, 1984.

Observed trait[a]		Anther culture line no.			Check varieties	
		608-1[b]	1166[c]	1166-4[c]	Setbyeolbyeo (susceptible)	Sangpungbyeo (resistant)
Discoloration[d]:	1	2	2	2	9	1
	2	4	5	6	6	2
Heading:	in (17 °C)	136	136	133	144	137
	out (25-27 °C)	109	116	118	127	128
Fertility (%):	in	7	53	51	0	8
	out	90	76	78	18	91
Spikelets (no.):	in	59	78	79	71	102
	out	140	130	77	132	101
Culm length (cm):	in	55	46	39	–	53
	out	87	54	53	–	69
Panicles (no.):	in	24.6	21.4	22.0	–	22.0
	out	23.2	22.2	22.8	–	20.6
Panicle exsertion[d]		4	6	6	9	5
Phenotypic acceptability[d]		5	4	5	9	5
Vigor[d]		1	1	1	7	5

[a] in = inlet temperature = 12 °C, out = outlet temperature = 25-27 °C. [b] Taipei 309/Tatsumi mochi. [c] Tatsumi mochi/BG90-2. [d] By Standard Evaluation System for Rice (7).

Table 8. Green plant production from the anther culture of 23 crosses, 1983 WS to end December 1985.

Cross	Green plant production	
	Total	With seeds
SR11191	24	15
SR11193	0	0
SR11194	0	0
SR11238	1	0
SR11242	0	0
SR11426	183	74
SR11428	47	15
SR11433	1110	493
SR11436	108	40
SR11440	19	3
SR11451	1548	663
SR11452	2641	938
SR11453	1807	915
SR11455	27	16
SR12192	235	88
SR12194	3	2
SR12196	150	55
SR12200	323	87
SR12211	156	54
SR12212	98	67
SR12246	190	92
SR12259	0	0
SR12291	0	0
Total	8670	3617
Percentage		41.7

Appr[illegible] media composition (mg/liter) of liquid E-24 and G4 for [illegible]s induction and semisolid N-19 for plant regeneration.

Component	E-24	G4	N-19
NH_4NO_3	–	–	16500
KNO_3	2500	2500	1900
$(NH_4)_2SO_4$	134.0	134.0	–
$MgSO_4 \cdot 7H_2O$	250.0	250.0	370.0
$MnSO_4 \cdot H_2O$	10.0	10.0	22.3
$NaH_2PO_4 \cdot H_2O$	150.0	150.0	–
$ZnSO_4 \cdot 7H_2O$	2.0	2.0	8.6
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CaCl_2 \cdot 2H_2O$	150.0	150.0	440.0
KI	0.75	0.75	0.83
H_3BO_3	3.0	3.0	6.2
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
KH_2PO_4	–	–	170.0
$NaMoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.85
Na_2EDTA	37.3	37.3	37.25
Nicotic acid	1.0	1.0	0.5
Thiamine HCl	10.0	10.0	0.1
Pyrodoxine HCl	1.0	1.0	0.5
Inositol	160	160	100
Glucose	5.0 g/liter	–	–
Glycine	–	–	2.0
Sucrose	20 g/liter	20 g/liter	30 g/liter
Agar	–	–	8.0 g/liter
Kinetin	–	–	1.0
NAA	–	–	1.0
2,4-D	1.0	1.0	–
BAP	0.5	0.5	–
IAA	0.5	0.5	–

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